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Antibody Responses to Hepatitis C Envelope Proteins in Patients With Acute or Chronic Hepatitis C

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Antibody responses to the hepatitis C virus (HCV) envelope proteins E1 and E2 were analyzed using two original assays in sera from 86 patients in different stages of disease. A Western blot assay and an immunofluorescence assay (IFA) were developed using envelope proteins produced, respectively, in *Escherichia coli* and in CV1 cells infected with a recombinant SV40. As a third method, the INNO-LIA HCV Ab III assay including E2 synthetic peptides was used. Of 38 chronically infected patients positive for anti-E2 antibodies by IFA, 26 were positive in the Western blot assay (68%) and 25 in the INNO-LIA test (66%). Thus, the detection of anti-envelope antibodies is highly dependent on the antigen formulation, and a native glycosylated form of the proteins is probably needed for their efficient detection. This study shows that the antibody response to HCV envelope proteins depends on the phase of infection. A few acutely infected patients displayed a response to E1 or E2 (36% by Western blot, 7% by IFA), and these antibodies seem to develop in patients evolving toward chronicity. The high prevalence in chronically infected subjects (62% to E2 by Western blot, 90% by IFA), particularly in subjects with essential mixed cryoglobulinemia (68% and 100%), confirms that the resolution of infection involves more than these antibodies. The antienvelope response in patients treated with interferon was investigated, but no significant relationship was found between antibody level prior to treatment and the evolution of hepatitis. The detection of anti-envelope antibodies, therefore, is not predictive of the response to antiviral therapy.

KEY WORDS: hepatitis C virus, envelope proteins, specific antibodies, infection stages, antigenic formulation

INTRODUCTION

Hepatitis C virus (HCV), the major cause of parenterally as well as sporadically transmitted non-A, non-B hepatitis [Kuo et al., 1989], is a small enveloped virus with a positive single-strand RNA genome encoding a large polyprotein of 3,011–3,033 amino acid residues [Choo et al., 1991; Takamisawa et al., 1991]. The capsid protein (C) and the two envelope glycoproteins (E1 and E2) are encoded by the N-terminal at one-fourth of the genome, whereas the remaining part of the genome codes for the nonstructural proteins NS2 to NS5B [Grakoui et al., 1993; Ralston et al., 1993].

Since the HCV genome has been cloned, several screening assays for HCV-specific antibodies have been developed. First-generation immunoassays were based on a recombinant protein (c100-3) expressed from the NS4 region [Kuo et al., 1989]; however, these assays yielded both false-positive and false-negative results [Colombo et al., 1989; Van der Poel et al., 1989]. To increase the sensitivity and specificity of the tests, both HCV structural and nonstructural proteins were used as target antigens in second-generation assays. These tests, which allowed the detection of antibodies against recombinant proteins from NS4 (c100-3), C (c22-3), and NS3 (c33c) regions, have exhibited higher sensitivity [Bresters et al., 1992; Katayama et al., 1992]. More recently, third-generation assays were developed including parts of C, NS3, NS4, and NS5 proteins [Vernelen et al., 1994]. Due to the improvement in the antigenicity of the NS3 and NS4 proteins, these tests have greatly ameliorated the sensitivity and specificity of HCV antibody detection [Pawlotsky et al., 1994; Uyttendaele et al., 1994]. The methods used to examine the antigen–antibody reactivity vary with the test (enzyme-linked assays, EIA or ELISA, or recombinant immunoblot assays) as well as

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the source of antigens (recombinant proteins or synthetic peptides), so sensitivities and specificities of the different tests have been reported to differ [Chiba et al., 1991; Ishida et al., 1993]. Amplification of HCV RNA by reverse transcription polymerase chain reaction (RT-PCR) has been proved to be a reliable and sensitive method to detect viral sequences in serum samples [Weiner et al., 1990]. However, this technique is of limited use in clinical practice because of economic considerations and problems of standardization. Specific problems remain to be solved in the diagnosis of HCV infections: (1) patients negative for anti-HCV antibodies by second- or third-generation diagnostic assays but positive for HCV RNA by RT-PCR, (2) early diagnosis of acute hepatitis C (anti-HCV antibodies are detected only late after onset of the disease), and (3) HCV variants escaping serodiagnosis and RT-PCR detection.

Little is known about the seroprevalence of antibodies (Ab) to the E1 and E2 glycoproteins during the different phases of hepatitis C infection. The frequency of Ab to E2, expressed as a fusion protein in *Escherichia coli,* has been reported to be as low as 20% in infected patients [Inoue et al., 1992; Mita et al., 1992; Yokosuka et al., 1992]. From a recent report, the detection of Ab to E1 and E2 seems to depend on the vector used for the expression of the protein [Hsu et al., 1993]. The frequency of Ab to glycosylated forms of the envelope proteins was found to be as high as 97% among HCV-infected patients with chronic liver disease [Chien et al., 1993] or patients with HCV RNA-positive plasmas [Lesniewski et al., 1995]. To clarify the role of anti-E1 and anti-E2 Ab during the course of HCV infection, we studied four different groups of patients using two originally developed assays. Namely, we examined evidence of antienvelope antibodies in acutely infected patients, subjects with recent hepatitis, and chronically infected patients with or without mixed cryoglobulinemia. Two detection assays were used systematically. Firstly, nonglycosylated E1 and E2 proteins were produced as fusion proteins in bacteria, and the reactivity of patients' sera against these antigens was analyzed by Western blot. Secondly, CV1 cells were infected with recombinant SV40 vectors expressing E1 or E2, and the infected cells were fixed and used to test the reactivity of patients' sera by an indirect immunofluorescence assay (IFA). As a third test method, the antienvelope response was analyzed by a third-generation immunoblot assay (INNO-LIA HCV Ab III), which includes E2 synthetic peptides. Subjects undergoing treatment with interferon alpha (IFN-α) were also included in the study to examine the possible relationship between prevalence of anti-E1 or E2 Ab and outcome of the treatment.

MATERIALS AND METHODS

Patients

Four groups of patients were included in this study.

Group I: Healthy control group (30 blood donors) selected according to conventional criteria: alanine aminotransferase (ALT) below the "upon limit of normal range" (ULNR) and negative for anti-HIV Ab, anti-HTLV Ab, HBs Ag, anti-HBc Ab, anti-HCV Ab, antimalarial Ab, and VDRL.

Group II: Patients with acute hepatitis C (n = 14), defined as subjects having acute ALT elevation after transfusion (ALT > 2 ULNR), with HCV RNA detectable at the onset of hepatitis and anti-HCV seroconversion a few days after the ALT peak. All patients were anti-HCV-negative before hepatitis and had no other cause of liver dysfunction. Serum samples were collected within a mean delay of 1.2 months after the acute phase. During the follow-up of patients, several samples were collected and examined.

Patients were included in groups III and IV according to the absence or the presence of cryoglobulins in their sera.

Group III: Patients with chronic hepatitis C (n = 23), community-acquired or with history of parenteral exposure, diagnosed as having ALT > 1.5 ULNR for longer than 6 months, anti-HCV Ab, liver biopsy findings compatible with chronic hepatitis [Knodell et al., 1981], and no other causes of liver dysfunction.

Group IV: Patients with chronic hepatitis C, presenting a symptom of essential mixed cryoglobulinemia (MC) (19 subjects, nine of whom had clinical symptoms related to cryoglobulinemia: vasculitis (three cases), peripheral neuropathy (five cases), arthralgia (two cases), or Raynaud phenomenum (one case). Eleven patients had type III MC and eight type II MC [Brouet et al., 1974]. Detection, isolation, and characterization of cryoglobulins were performed as described [Musset et al., 1992].

All patients in groups III and IV were treated with IFN-α 2a or 2b after diagnosis (3 MU to 6 MU three times a week for 6 months). After 6 months, IFN-α treatment was stopped and patients were classified as either responders (ALT ≤ ULNR at the end of the treatment) or nonresponders (ALT > ULNR at the end of the treatment) on the basis of serum ALT levels. Moreover, all responders were scored for viremia by RT-PCR and found negative 6 months after the treatment.

Serum samples obtained 1 year after the first sample were analyzed from seven patients of group I. These samples were gathered with those from nine patients of group II who had chronic hepatitis for less than 2 years, and these 16 samples were considered as the "recent hepatitis group."

Anti-HCV Ab were assayed for all sera using third-generation ELISAs: Ortho HCV ELISA (Ortho Diagnostic Systems, Chiron Corp., Emeryville, CA) and Monolisa (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) according to the protocol provided by the manufacturer. Repeatedly reactive sera were confirmed using two third-generation RIAs (CHIRON RIBA HCV, Ortho Diagnostic Systems, and INNO-LIA HCV Ab III, Innogenetics, Ghent, Belgium).

Viral RNA was extracted from serum samples, reverse-transcribed, and amplified by nested PCR with primers located in the highly conserved 5′ terminal noncoding sequence of the genome as described [Lunel et al., 1994]. All of the samples were also genotyped after amplifica-

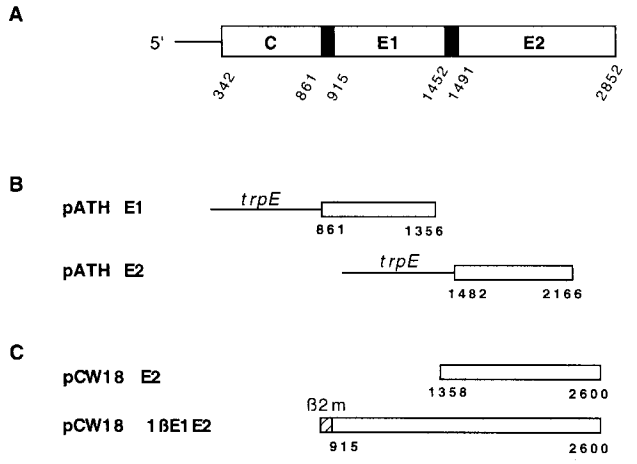

Fig. 1. Schematic representation of the HCV structural genes cloned into expression plasmids. **A:** Diagram of the 5′ region of the HCV-H strain genome showing the 5′ noncoding sequence and the three structural proteins with their signal peptide (black boxes). **B:** HCV sequences expressed as *trpE* fusion proteins in pATH bacterial expression vectors. The amino terminal extremity of the HCV sequence was fused in frame to the amino terminal two-thirds of the *trpE* gene of *E. coli*. **C:** HCV sequences expressed with recombinant SV40. HCV sequences were inserted downstream from the ATG of the SV40 VP1 gene into an SV40 genome. For the pCW18 1βE1E2 construct, the E1 gene was cloned in frame downstream from the signal peptide of the β2 microglobulin (hatched box). Numbers refer to nucleotide position on the HCV-H genome map.

tion using the line probe assay (LiPA; Innogenetics) [Stuyver et al., 1993]. This assay allows typing and subtyping of the most common HCV genotypes and subtypes (1a, 1b, 2a, 2b, 3a, 4, and 5).

Differences in proportions were tested for significance using Fischer's test. A P value < 0.05 was considered significant.

Bacterial Expression of HCV Proteins E1 and E2

HCV E1 and E2 antigens were produced as fusion proteins using a pATH vector [Reef Hardy and Strauss, 1988]. HCV DNA fragments 861 to 1356 and 1482 to 2166, coding for amino acid residues 174 to 338 from E1 and 381 to 610 from E2, respectively, were PCR-amplified from a full-length HCV strain H cDNA (genotype 1a) (Fig. 1). The DNA fragment coding for E1, made blunt at its 5′ terminus and digested with *Bam*H1 at the 3′ terminus, was inserted into the pATH20 vector digested with *Sma*I and *Bam*HI to generate plasmid pATH E1. The DNA fragment coding for E2 was digested with *Hinc*II and inserted into the pATH2 vector digested with *Sma*I to generate pATH E2. Resulting plasmids were used to transform *E. coli* and to prepare bacterial extracts containing *trpE*-E1 or *trpE*-E2 as described [Cahour et al., 1992]. These were used to perform Western blot and to immunize rabbits as described [Cahour et al., 1992]; rabbit anti-sera have been described previously [Fournillier-Jacob et al., 1996].

Mammalian Cell Expression of HCV Proteins E1 and E2

Mammalian expression vectors were derived from the simian SV40 vectors pCW18 PL1 [Despres et al., 1988] and pCW18 1β2m [Wychowski et al., unpublished results]. Plasmid pCW18 PL1 contains an SV40 genome deleted of the VP1 sequence in which a polycloning site has been placed immediately downstream from the initiator ATG of the SV40 VP1 gene. Plasmid pCW18 1β2m contains, between the SV40 VP1 ATG and the cloning site, the sequence coding for the β2 microglobulin protein and its signal peptide.

The cDNA fragments corresponding to HCV sequences 1358 to 2600 and 915 to 2600 were PCR-amplified and filled in with the Klenow enzyme (Fig. 1). The first fragment was inserted into the unique *Eco*R1 site in the pCW18 PL1 vector polylinker made blunt. The resulting plasmid, designated pCW18 E2, encodes the E2 protein preceded by its signal peptide and placed under the control of the SV40 late transcription promotor. The second cDNA fragment, coding for E1 and E2 proteins, was inserted into the pCW18 1β2m vector between the blunted *Acc*I and *Bgl*II sites to generate the recombinant plasmid pCW18 1βE1E2. In that construct, the putative signal peptide of E1 was replaced by that of the β2 microglobulin protein.

Preparation of DNAs used for the production of recombinant SV40 SV/E2 and SV/1βE1E2 was performed as described [Wychowski et al., 1986]. Generation of SV40-HCV recombinants was performed on CV1 cells maintained in Dulbecco's modified Eagle's essential minimal medium (DMEM) (Gibco BRL, Eragny, France) supplemented with 10% tryptose phosphate, 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at 10^6 cells per dish and cotransfected with 0.5 μg DNA from recombinant pCW18 1βE1E2 or pCW18 E2 and 0.25 μg DNA from helper SV40 mutant am404, as described [Wychowski et al., 1986]. Mixed viral stocks, called SV/E2 or SV/1βE1E2, were produced and used to express HCV envelope proteins.

Western Blot Analysis

Crude extracts containing fusion proteins E1 or E2 were fractionated on 8% SDS-PAGE and then electrotransferred to a nitrocellulose membrane (Hybond-C; Amersham, Les Ulis, France) in transfer buffer (50 mM Tris base, 50 mM boric acid). After incubation for 10 min at room temperature in buffer 1 (20 mM Tris HCl, pH 7.5, 500 mM NaCl), the membrane was incubated overnight at 4°C in buffer S (buffer 1 plus 0.1% Tween 20, 1% BSA) containing the patient serum at a 1:300 dilution. After three washes in buffer 1, the membrane was incubated for 1 hr with horseradish peroxidase-conjugated goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL) at a 1:500 dilution. The blot was washed again and staining was developed with 4-chloro-1-naphtol substrate (Sigma, St Louis, MO).

Indirect Immunofluorescence Assays

CV1 cells, seeded on coverslips at 10^5 cells per dish, were infected 24 hr later with SV40-HCV recombinants at a multiplicity of infection of 5 PFU/cell. Forty-five hours postinfection, infected cells were washed with PBS (phosphate-buffered saline) and fixed for 10 min at −20°C in methanol-acetone (7/3: v/v). Coverslips were then incubated for 30 min with diluted patient sera (1:20 in PBS or in PBS/0.7% β-mercaptoethanol) or rabbit antisera (1:25 in PBS). After washes, coverslips were incubated for another 30 min with a 1:400 dilution of fluorescein-conjugated goat anti-human or anti-rabbit IgG (Biomérieux, Marcy-l'Etoile, France). After washes, the coverslips were mounted for microscopy examination. The 1:20 dilution was chosen as a standard, evaluated by testing different dilutions of several patient sera (1:5, 1:10, 1:20, 1:50, 1:100).

RESULTS

Expression of HCV E1 and E2 Envelope Proteins

Two systems were used to generate HCV envelope proteins E1 and E2: a bacterial expression system based on the production *trpE* fusion proteins and a mammalian cell expression system based on recombinant SV40 vectors (Fig. 1). The specific recognition of E1 and E2 proteins was analyzed with anti-E1 and anti-E2 rabbit sera and with serum samples from a healthy HCV-negative subject and a chronically HCV-infected patient with HCV antibodies and HCV RNA.

The crude extract of *E. coli* expressing *trpE*-E1 or *trpE*-E2 fusion proteins was separated by 8% SDS-PAGE prior to staining with Coomassie blue. The apparent molecular weights of the E1 and E2 proteins, fused in frame with a part of the *trpE* polypeptide, were 52 and 62 kDa, respectively (Fig. 2A). Characterization of these proteins was also performed by Western blot using human serum samples (Fig. 2B). Prominent bands in the predicted positions of about 52 and 62 kDa appeared only with the serum from the HCV-infected patient, indicating that these bands were HCV-specific.

The HCV E1 and E2 glycoproteins produced with recombinant SV40 SV/E2 and SV/1βE1E2 were characterized by IFA using the same human samples (Fig. 3). The fluorescent staining pattern, observed only with the serum sample from the HCV-infected patient, showed accumulation of the antigen in the cytoplasm, as was previously observed with a recombinant vaccinia virus system [Selby et al., 1993].

Antibody Response to HCV Envelope Proteins at Different Stages of the Hepatitis

Reactivity of all the patients' sera against the HCV envelope proteins was examined in parallel by Western blot with *trpE*-E1 and *trpE*-E2, by IFA with SV/1βE1E2- and SV/E2-infected cells, and by INNO-LIA HCV Ab III assay with E2 peptides. Each analysis included positive and negative serum controls. For Western blot and INNO-LIA assays, the positive and negative serum controls were used for the characterization of the *trpE* fusion proteins. For IFA analysis, we used the same positive control; the negative control corresponded to wild-type SV40-infected cells. All serum samples were repeatedly tested by the three assays.

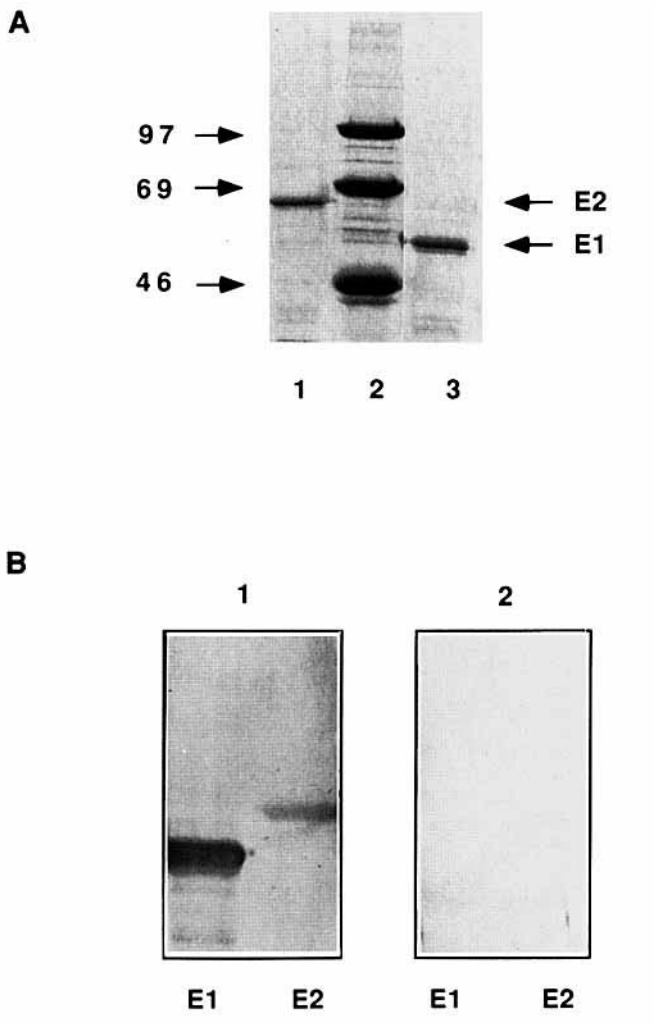

Fig. 2. Expression of HCV envelope proteins as *trpE* fusion proteins. **A:** Coomassie blue-stained gel after SDS-PAGE showing E2 **(lane 1)** and E1 **(lane 3)** as *trpE* fusion proteins (arrows). Positions of molecular weight markers shown in **lane 2. B:** Western blot analysis of *trpE*-envelope proteins E1 and E2 with serum samples from a chronically HCV-infected patient (strip 1) and a healthy subject (strip 2).

Serum samples from group I (healthy subjects) remained negative for anti-envelope protein antibodies with the three methods.

For group II (acute hepatitis C), five of 14 samples reacted with *trpE*-E1 (36%) and four of 14 with *trpE*-E2 (29%) by Western blot. Only one of the 14 samples was positive with E2 and with E1 and E2 by IFA(7%). All samples remained negative for anti-E2 antibodies by INNO-LIA assay (Table I).

To analyze reactivity to the envelope proteins of patients' sera with recent hepatitis, serum samples obtained 1 year after the first sample were examined from

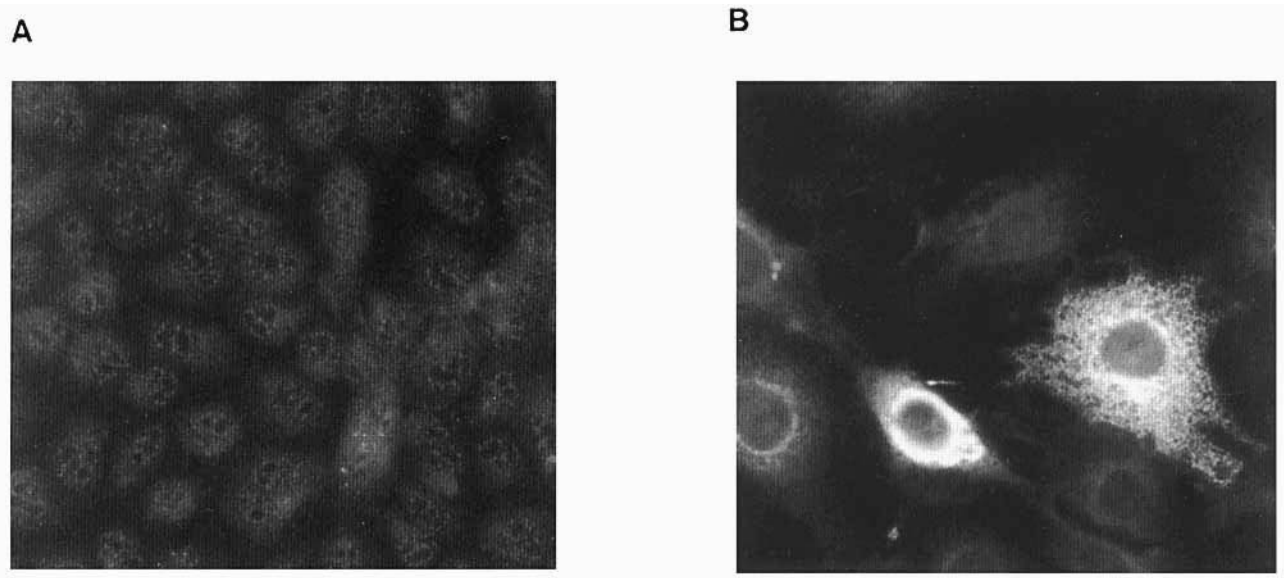

Fig. 3. Indirect immunofluorescence analysis of CV1 cells infected with the SV40-E2 recombinant using serum samples (1:20 dilution) from a healthy subject **A** or a chronically HCV-infected patient **B.** Staining was with a goat anti-human IgG immune serum coupled with fluorescein.

TABLE I. Serum Reactivity to HCV Envelope Proteins in Patients With Acute Hepatitis

	Reactivity				
Patients	*trpE*-E1	*trpE*-E2	SV/E2	SV/1βE1E2	E2 peptides
A	−	−	−	−	−
B	−	−	−	−	−
C	−	−	−	−	−
D	−	−	−	−	−
E	−	−	−	−	−
F	+	+	−	−	−
G	−	−	−	−	−
H	+	−	−	−	−
I	+	−	−	−	−
J	−	−	−	−	−
K	+	+	−	−	−
L	−	+	−	−	−
M	−	−	−	−	−
N	+	+	+	+	−
Total: 14	5 (36%)[a]	4 (29%)	1 (7%)	1 (7%)	0 (0%)

Assays were as described in "Materials and Methods" using either Western blot (*trpE*-E1 and *trpE*-E2), IFA (SV/E2 or SV/βE1E2), or immunoblot (E2 peptides).
[a](X%): Cumulative percent seropositive.

seven patients of group II. These samples were gathered with those from nine patients of group III who had chronic hepatitis for less than 2 years, and these 16 samples were considered as the "recent hepatitis group." Among the 16 patients of this group, 11 displayed reactivity to *trpE*-E1 (69%), 12 to *trpE*-E2 (75%), 12 to E2 expressed in mammalian cells (75%), 11 to E1 and E2 coexpressed in mammalian cells (69%), and four to E2 peptides (25%) (Table II). In this group, no difference was found between our two methods in the efficiency of E2 detection. In contrast, the INNO-LIA assay seemed to display a lower efficiency ($P = 0.006$).

Of the 23 patients of group III (chronic hepatitis C), 13 were positive for *trpE*-E1 and *trpE*-E2 by Western blot (57%), 19 for E2 or E1 and E2 by IFA (83%), and 16 for E2 peptides (70%). The reactivity of group III sera against E2 expressed in mammalian cells and analyzed by IFA was significantly higher than that observed against the *trpE* fusion protein ($P = 0.04$). The results obtained with the INNO-LIA assay were similar in terms

TABLE II. Serum Reactivity to HCV Envelope Proteins in Patients With Recent Hepatitis, Chronic Hepatitis (III), and Chronic Hepatitis With Cryoglobulinemia (IV)

Group	Number of patients	Reactivity				
		trpE-E1	*trpE*-E2	SV/E2	SV/1βE1E2	E2 peptides
Recent hepatitis	16	11 (69%)	12 (75%)	12 (75%)	11 (69%)	4 (25%)
III	23	13 (57%)	13 (57%)	19 (83%)	19 (83%)	16 (70%)
IV	19	14 (74%)[a]	13 (68%)	19 (100%)	18 (95%)	9 (47%)

[a](X%): Cumulative percent seropositive.

of efficiency. Positive results obtained with the *trpE*-E2 protein were concordant with those obtained with the E2 protein expressed in mammalian cells in 85% of serum samples. Between the INNO-LIA E2 peptides and the E2 protein expressed in mammalian cells, positive results were concordant in 75% of the cases.

Detection of Anti-HCV Envelope Protein Antibodies Among HCV-Infected Patients With Cryoglobulinemia

We focused our attention on chronically HCV-infected patients with essential mixed cryoglobulinemia (group IV), a symptom frequently associated with hepatitis C [Lunel et al., 1994]. Our aim was to define if patients with MC could present a different reactivity against envelope proteins than patients without this symptom. Among the 19 patients of group IV, 14 displayed reactivity to *trpE*-E1 (74%), 13 to *trpE*-E2 (68%), 19 to E2 expressed in mammalian cells (100%), 18 to E1 and E2 coexpressed in mammalian cells (95%), and nine to E2 peptides (47%) (Table II). Reactivity of the group IV sera with E2 expressed in mammalian cells was significantly higher than that with the *trpE*-E2 product ($P = 0.01$) or with E2 peptides ($P = 0.0002$). Samples positive for the *trpE*-E2 protein were also positive for E2 expressed in mammalian cells; there was also concordance between results obtained using the INNO-LIA E2 peptides and E2 expressed in mammalian cells.

Correlation Between Anti-HCV Envelope Antibodies and the Response to Interferon Therapy

It was of interest to determine if a correlation might exist between the occurrence of antienvelope Ab prior to interferon therapy and the evolution of hepatitis after treatment. All 42 patients in groups III and IV were treated after diagnosis with 3–6 MU interferon three times a week for 6 months. Ten responded to the treatment (R), 16 responded with relapse upon cessation of the treatment (RR), and 16 were nonresponders (NR). Genotyping of the viruses confirmed that patients infected with genotype 2a viruses (n = 9) showed the best response to the therapy (44% were responders), whereas those infected with genotype 1b (n = 17) were the worst responders (12% R) [Takada et al., 1992]. The antibody response to the HCV envelope proteins was measured by IFA before the onset of interferon treatment. As shown in Table III, there was no significant difference in the prevalence of anti-HCV envelope Ab between the responder patients and a group corresponding only to the RR and NR patients. We also analyzed, 6 months after cessation of the interferon treatment, serum provided by one responder, one partial responder, and one nonresponder who were positive for antienvelope Ab at the onset of treatment. All of them were positive by IFA for the HCV envelope proteins in spite of the different evolution of their diseases. Altogether, these data suggest that the detection of antienvelope Ab may not be predictive of the response to antiviral therapy.

DISCUSSION

In spite of the development of three successive generations of tests to detect HCV antibodies, problems remain with the serodiagnosis of hepatitis C. In particular, the diagnosis of early acute hepatitis C is difficult [Yeh et al., 1994] and indeterminate results are found with patients suspected to have progressive viral liver disease [Garcia-Samaniego et al., 1993]. The E1 and E2 envelope glycoproteins are obvious targets for the immune response to HCV infection. However, these proteins are not included in current generation HCV screening tests partly because of the difficulties and high cost associated with purification and manufacture of them. To clarify the role of anti-E1 and anti-E2 antibodies during the course of HCV infection, the specific antibody responses to E1 and E2 in different groups of patients were investigated. To diversify the test methods used, E1 and E2 were expressed with prokaryotic or eukaryotic expression systems and the antibody response was detected by Western blot or IFA, respectively. In addition, the anti-E2 response was examined using the third-generation immunoblot assay INNO-LIA.

The results show that the detection of antienvelope antibodies is highly dependent on the antigen production method chosen and the test method used. Of 38 chronically infected patients positive for antibodies to E2 by the IFA test, only 26 were positive by Western blot for *trpE*-E2 (68%) and 25 reacted to the E2 peptides of the INNO-LIA assay (66%). These different results might be explained by the fact that HCV envelope proteins, which contain multiple glycosylation sites, are efficiently glycosylated when expressed in mammalian cells and are analyzed under a more native form when visualized by IFA. In contrast, proteins used in the form of peptides or expressed in *E. coli* are unglycosylated. Moreover, the antigenicity of the envelope proteins might be affected by the fusion with *trpE* protein, although the fusion proteins are denatured in a Western blot. In addition,

TABLE III. Serum Reactivity to HCV Envelope Proteins in Patients With Chronic Hepatitis Treated With Interferon

Group	Number of patients	Reactivity	
		SV/E2	SV/1βE1E2
R[a]	10	8 (80%)[b]	8 (80%)
RR and NR	32	30 (94%)	29 (91%)
Overall	42	38 (90%)	37 (88%)

[a]Responders to treatment. RR: Patients relapsing upon cessation of treatment. NR: Nonresponders.
[b](X%): Cumulative percent seropositive.

analysis of the proteins by Western blot results in the destruction of conformational epitopes, which are also absent in the INNO-LIA immunoblot test. As previously described [Chien et al., 1993], these data indicate that antibodies to both linear and conformational epitopes of E2 are elicited during chronic HCV infections. Expression of HCV-glycosylated envelope proteins and analysis of their antigenicity in a native form seems to be required for the efficient detection of antienvelope antibodies in patients' sera.

This study showed that the antibody response to HCV envelope proteins is highly dependent on the phase of infection. A few patients with acute hepatitis C displayed a response to the bacterially expressed E1 and E2 products (29% to E2, 36% to E1) but only one of 14 (7%) to the envelope proteins expressed in mammalian cells and none to the peptides of the INNO-LIA test. Follow-up of patients with recent hepatitis showed that seroreactivity against the HCV envelope proteins increased with time to high figures (69% to E1 and 75% to E2 by Western blot, 75% to E2 and 69% to E1E2 by IFA), although the percentage of reactivity remained low (25%) when assessed by the INNO-LIA assay. The prevalence of antienvelope antibodies was the highest among chronically infected patients of groups III and IV (64% to E1 and 62% to E2 by Western blot; 90% to E2, 88% to E1E2, and 60% to E2 peptides by IFA). These results suggest that the response against the HCV envelope proteins occurs only late after the onset of hepatitis and develops mostly in the chronic stage of the disease. However, Lesniewski et al. [1995] have shown that HCV-seropositive plasma donors reacted with E2 early after the first ALT elevation using an enzyme immunoassay. This discrepancy might be due again to differences in antigens and their methods of production. Our data concerning patients with recent hepatitis show that antibodies specific to the envelope proteins seem to develop in HCV-infected patients evolving toward chronicity. At this stage of the disease, antibodies to E1 or E2 seem to be markers as efficient as the antibodies to other HCV proteins included in commercial HCV blood screening tests. The persistence of chronic infection in spite of the presence of these antibodies confirms that the resolution of infection involves more than the simple presence of envelope-targeted antibodies [Chien et al., 1993; Hsu et al., 1993; Mink et al., 1994].

The prevalence of antienvelope antibodies was higher in chronically infected patients with MC than in those without MC. However, a wider set of samples needs to be analyzed to make the difference significant. MC is found in 50% of HCV-infected patients [Lunel et al., 1994], but the role of HCV in the pathogenesis of cryoglobulinemia is unclear. Multiple factors appear to be responsible for the production of cryoglobulins in HCV-infected patients, including cirrhosis, duration of liver disease, and viral infection itself, which may act directly or through the host immune response it elicits [Lunel, 1994]. Agnello et al. [1992] observed that HCV antibodies and HCV RNA were concentrated in cryoglobulins. These observations together with the disappearance or the decrease of cryoglobulins during IFN therapy [Misiani et al., 1993] also support the hypothesis of a direct role of HCV in cryoglobulin production. Cryoglobulins may be made of HCV antigen–antibody complexes and could be one way for trapping the virus. It should be noticed that the antienvelope antibodies were not concentrated in the cryoprecipitate as could have been expected. Indeed, no difference was observed between the reactivity to E1 and E2 of the cryoprecipitate and supernatant at different dilutions of the sera from two patients of group IV (data not shown).

Although the presence of antibodies to E1 and E2 is not necessarily associated with viral clearance during the course of HCV infection, we investigated the possible correlation between the response to IFN therapy and the humoral response to HCV envelope proteins prior to treatment. There was unfortunately no detectable relationship between the presence of antibodies to HCV envelope proteins before treatment and the evolution of hepatitis after treatment. Detection of these antibodies does not, therefore, seem to provide information for prognosis of the outcome of the disease after IFN therapy. Maertens et al. [1994] have shown that anti-E1 reactivity decreased significantly in patients with persistent ALT normalization after treatment. In the limited set of three sera examined here at 6 months after cessation of the IFN treatment, antienvelope antibodies seemed to persist independently of the response of the patient to the antiviral therapy. To determine if the decrease of antienvelope antibodies under IFN is a predictive marker of a sustained response to treatment, further studies should be done including more patients and a longer follow-up after cessation of the treatment.

In conclusion, the antibody response to HCV envelope proteins depends on the phase of infection. Using three different assays, it was observed that the antibody response against HCV envelope proteins is difficult to detect during the acute phase of hepatitis C but correlates

with evolution of the disease to chronicity. Moreover, analysis of the antienvelope response with different antigens and different test methods demonstrated that the detection of anti-E1 and anti-E2 antibodies is highly dependent on the antigenic formulation. Depending on the antigen production method chosen and the test method used, the usefulness of E1 and E2 antibodies for the diagnosis of different stages of hepatitis will vary significantly.

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REFERENCES

Agnello V, Chung RT, Kaplan LM (1992): A role for hepatitis C virus infection in type II cryoglobulinemia. New England Journal of Medicine 327:1490–1495.

Bresters D, Cuypers HTM, Reesink HW, Schaasberg WP, van der Poel CL, Mauser-Bunschoten EP, Houghton M, Choo Q-L, Kuo G, Lesniewski R, Troonen H, Lelie PN (1992): Enhanced sensitivity of a second generation ELISA for antibody to hepatitis C virus. Vox Sanguinis 62:213–217.

Brouet JC, Clauvel JP, Klein M, Seligman M (1974): Biological and clinical significance of cryoglobulins. A report of 86 cases. American Journal of Medicine 57:775–788.

Cahour A, Falgout B, Lai C-J (1992): Cleavage of the Dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. Journal of Virology 66:1535–1542.

Chiba J, Ohba H, Matsuura Y, Watanabe Y, Katayama T, Kikuchi S, Saito I, Miyamura T (1991): Serodiagnosis of hepatitis C virus (HCV) infection with an HCV core protein molecularly expressed by a recombinant baculovirus. Proceedings of the National Academy of Sciences, USA 88:4641–4645.

Chien DY, Choo Q-L, Ralson R, Spaete R, Tong M, Houghton M, Kuo G (1993): Persistence of HCV despite antibodies to both putative envelope glycoproteins. Lancet 342:933

Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby A, Barr PJ, Weiner AJ, Bradley DW, Kuo G, Houghton M (1991): Genetic organisation and diversity of the hepatitis C virus. Proceedings of the National Academy of Sciences, USA 88:2451–2455

Colombo M, Kuo G, Choo Q-L, Donato MF, Del Ninno E, Tommasini MA, Dioguardi N, Houghton M (1989): Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. Lancet 2:1006–1008.

Despres P, Cahour A, Wychowski C, Girard M, Bouloy M (1988): Expression of the yellow fever virus envelope protein using hybrid SV40/yellow fever viruses. Annales de l'Institut Pasteur/Virologie 139:59–67.

Fournillier-Jacob A, Cahour A, Escriou N, Girard M, Wychowski C (1996): Processing of the E1 glycoprotein of hepatitis C virus expressed in mammalina cells. Journal of General Virology, (1996) 77:1055–1064.

Garcia-Samaniego J, Enriquez A, Soriano V, Gutierrez M, Baquero M, Munoz F (1993): Third-generation recombinant immunoblot assay to confirm hepatitis C virus-indeterminate serological samples. Vox Sanguinis 64:191–192.

Grakoui A, Wychowski C, Lin C, Feinstone SM, Rice CM (1993): Expression and identification of hepatitis C virus polyprotein cleavage products. Journal of Virology 67:1385–1395.

Hsu HH, Donets M, Greenberg H, Feinstone SM (1993): Characterization of hepatitis C virus structural proteins with a recombinant baculovirus expression system. Hepatology 17:763–771.

Inoue Y, Suzuki R, Matsuura Y, Harada S, Chiba J, Watanabe Y, Saito I, Miyamura T (1992): Expression of the amino-terminal half of the NS1 region of the hepatitis C virus genome and detection of an antibody to the expressed protein in patients with liver diseases. Journal of General Virology 73:2151–2154.

Ishida C, Matsumoto K, Fukada K, Matsuhita K, Shiraki H, Maeda Y (1993): Detection of antibodies to hepatitis C virus (HCV) structural proteins in anti-HCV-positive sera by an enzyme-linked immunosorbent assay using synthetic peptides as antigens. Journal of Clinical Microbiology 31:936–940.

Katayama T, Mazda T, Kikuchi S, Harada S, Matsuura Y, Chiba J, Ohba H, Saito I, Miyamura T (1992): Improved serodiagnosis of non-A, non-B hepatitis by an assay detecting antibody to hepatitis C virus core antigen. Hepatology 15:391–394.

Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wollman J (1981): Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 5:431–435.

Kuo G, Choo Q-L, Alter HJ, Gitnick GL, Redecker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo M, Lee WS, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW, Houghton M (1989): An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364.

Lesniewski R, Okasinki G, Carrick R, Van Sant C, Desai S, Johnson R, Scheffel J, Moore B, Mushahwar I (1995): Antibody to hepatitis C virus second envelope (HCV-E2) glycoprotein: A new marker of HCV infection closely associated with viremia. Journal of Medical Virology 45:415–422.

Lunel F (1994): Hepatitis C virus and autoimmunity: Fortuitous association or reality? Gastroenterology 106:1291–1300.

Lunel F, Musset L, Cacoub P, Frangeul L, Cresta P, Perrin M, Grippon P, Hoang C, Piette JC, Huraux JM, Opolon P (1994): Cryoglobulinemia in chronic liver diseases—Role of Hepatitis C virus and liver damage. Gastroenterology 106:1291–1300.

Maertens G, Ducatteeuw A, Barlet V, Zarski JP, Vandeponseele P, Bosman F, de Martynoff G, Venneman G, Wyseur A, Stuyver L, Brouwer J, Heijtink R (1994): Decrease of anti-E1 antibody levels during monitoring of IFN-α therapy correlates with long-term, sustained response to treatment. Abstract presented at the 2nd International Meeting on Hepatitis C and Related Viruses. San Diego: University of California, p 265.

Mink MA, Benichou S, Madaule P, Tiollais P, Prince AM, Inchauspe G (1994): Characterization and mapping of a B-cell immunogenic domain in hepatitis C virus E2 glycoprotein using a yeast peptide library. Virology 200:246–255.

Misiani R, Bellavita P, Riuniti O (1993): Hepatitis C virus and cryoglobulinemia. New England Journal of Medicine 15:1121–1122.

Mita E, Hayashi N, Ueda K, Kasahara A, Fusamoto H, Takamizawa A, Matsubara K, Okayama H, Kamada T (1992): Expression of MBP-HCV NS1/E2 fusion protein in *E. coli* and detection of anti-NS1/E2 antibody in type C chronic liver disease. Biochemical Biophysical Research Communication 183:925–930.

Musset L, Diemert MC, Taibi F, Thi Huong Du L, Cacoub P, Leger JM, Boissy G, Gaillard O, Galli J (1992): Characterization of cryoglobulins by immunoblotting. Clinical Chemistry 38:798–802.

Pawlotsky JM, Fleury A, Choukroun V, Deforges L, Roudotthoraval F, Aumont P, Duval J, Dhumeaux D (1994): Significance of highly positive c22-3 indeterminate second-generation hepatitis C virus (HCV) recombinant immunoblot assay (RIBA) and resolution by third-generation HCV RIBA. Journal of Clinical Microbiology 32:1357–1359.

Ralston R, Thudium K, Berger K, Kuo C, Gervase B, Hall J, Selby M, Kuo G, Houghton M, Choo QL (1993): Characterization of hepatitis-C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. Journal of Virology 67:6753–6761.

Reef Hardy W, Strauss JH (1988): Processing the non-structural polyproteins of Sindbis virus: Study of the kinetics in vivo by using monospecific antibodies. Journal of Virology 62:998–1007.

Selby MJ, Choo Q-L, Berger K, Kuo G, Glazer E, Eckart M, Lee C, Chien D, Kuo C, Houghton M (1993): Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome. Journal of General Virology 74:1103–1113.

Stuyver L, Rossau R, Wyseur A, Duhamel M, Vanderborght B, Van Heuverswyn H, Maertens G (1993): Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. Journal of General Virology 74:1093–1102.

Takada N, Takase S, Takada A, Date T (1992): HCV genotypes in different countries. Lancet 339:808.

Takamisawa A, Mori C, Fuke I, Manabe S, Murakami S, Jujita J,

Onishi E, Andoh T, Yoshida I, Okayama H (1991): Structure and organisation of the hepatitis C virus genome isolated from human carriers. Journal of Virology 65:1105–1113.

Uyttendaele S, Clayes H, Meertens W, Verhaert H, Vermylen C (1994): Evaluation of third-generation screening and confirmatory assays for HCV antibodies. Vox Sanguinis 66:122–129.

Van der Poel CL, Reesink HW, Lelie PN, Leentvaar-Kuypers A, Choo Q-L, Kuo G, Houghton M (1989): Anti-hepatitis C antibodies and non-A, non-B post-transfusion hepatitis in the Netherlands. Lancet 2:297–298.

Vernelen K, Clayes H, Verhaert A, Volckaerets A, Vemylen C (1994): Significance of NS3 and NS5 antigens in screening for HCV antibodies. Lancet 343:853.

Weiner AJ, Kuo G, Bradley DW, Bonino F, Saracco G, Lee C, Rosenblatt J, Choo Q-L, Houghton M (1990): Detection of hepatitis C viral sequences in non-A, non-B hepatitis. Lancet 335:1–3.

Wychowski C, Benichou D, Girard M (1986): A domain of SV40 capsid polypeptide VP1 that specifies migration into the cell nucleus. EMBO Journal 5:2569–2576.

Yeh C-T, Han C-M, Lo S-Y, Ou J-H, Fan K-D, Sheen I-S, Chu C-M, Liaw Y-F (1994): Early detection of anti-HCc antibody in acute hepatitis C virus (HCV) by Western-blot (immunoblot) using a recombinant HCV core protein fragment. Journal of Clinical Microbiology 32:2235–2241.

Yokosuka O, Ito Y, Imazeki F, Ohto M, Omata M (1992): Detection of antibody to hepatitis-C E2/NS1 protein in patients with type C hepatitis. Biochemical Biophysical Research Communication 189:565–571.